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Donor Polymorphisms of TLR4 Associated with Graft Failure in Liver Transplant Recipients

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Abstract

There have been many reports showing significant associations between recipient genetic variants and allograft outcome, including acute rejection and graft failure, but less is known about the contribution of the donor genotypes. We analyzed 37 single nucleotide polymorphisms (SNPs) within the toll-like receptor 4 (TLR4) gene from deceased donor liver allografts, transplanted into 738 recipients, to determine their effects on liver graft failure (LGF). Two SNPs exhibited a significant association with LGF, rs11536865 (HR=2.5, p=0.0003) and rs5030717 (HR=1.67, p=0.00079), after adjusting for donor and recipient race and correcting for multiple test comparisons. An additional SNP, rs913930, exhibited a significant association in Caucasian donors (HR 1.62, p=0.00055) and two SNPs exhibited a suggestive association in African American donors, rs11536865 (HR=2.45, p=0.00205) and rs5030717 (HR=2.32, p=0.0024). Additionally, the Liver Donor Risk Index (HR 2.56, 95% CI=(1.54, 4.26, p=0.00031) and hepatitis C virus (HCV) status (HR=1.53, 95% CI=(1.04, 2.24), p=0.032) increased the risk of all-cause LGF in a Cox proportional hazards model adjusting for recipient race. Donor polymorphisms in TLR4 could be an important factor in modulating TLR4 activity and therefore affect the risk of graft loss. Additionally, there is a suggestion for an interaction between polymorphisms within TLR4 and HCV status.

Keywords

TLR4; donor; SNPs; genotyping; HCV; liver transplant

Introduction

There have been many attempts to associate solid organ allograft outcomes with specific genetic variants, usually in the form of single nucleotide polymorphisms (SNPs). Most studies have focused on recipient genotypes, but there is evidence that donor associated

genetic variants also play a role in affecting allograft outcome. For example, donor variation in the nuclear pregnane X receptor (PXR/NR1I2) has been associated with delayed graft function after renal transplantation (1), and donor variants of mannose binding lectin (MBL) and donor heme oxygenase-1 (HMOX1) have been associated with liver allograft outcomes including graft survival (2, 3).

Several recipient SNPs within the toll-like receptor 4 (TLR4) gene have been reported to be associated with kidney allograft dysfunction (4, 5). Additionally, variants in TLR4 have also been associated with ischemia and reperfusion injury in liver allografts and TLR4 signaling has been associated with acute rejection in the liver (6, 7). The TLR4 gene product plays a critical role in activating immune responses to bacterial infection. TLR4 is responsible for recognizing lipopolysaccharide (LPS) from gram-negative bacteria and inducing an inflammatory response resulting in the production of several pro-inflammatory, antiviral and anti-bacterial cytokines (8, 9). In healthy liver, the expression level of TLRs is low, but in the presence of LPS or liver injury, such as alcoholic liver disease or cirrhosis, expression of TLR4 increases (9–12). It has been shown that increased TLR4 expression in the liver is associated with hepatic inflammation and liver injury (13, 14). Increased TLR4 expression has also been hypothesized to be associated with acute rejection in liver allografts (15, 16). Increased TLR4 expression in kidney allografts has also been associated with acute rejection and chronic allograft nephropathy (17). The presence of the C/C genotype of TLR4 promoter SNP rs10759932 in either the recipient or the donor was associated with rejection free survival in kidney transplantation (5). Two common coding polymorphisms, rs4986790 (p.Asp299Gly) and rs4986791 (p.Thr399Ile) within the extracellular domain, that have been shown to attenuate TNF- α secretion, have been associated with reduced renal allograft rejection (18–20). The incidence of acute rejection in kidney allograft recipients was significantly reduced when donor genotypes were heterozygous for either variant (21).

We have previously reported that the TLR4 variant p.Asp299Gly loss-of-function mutation (rs4986790) in the donor liver attenuates the rate of post-ischemic acute liver injury but the relevance of donor-associated TLR4 activation on long-term liver function is unclear (4). We hypothesized that variants which alter TLR4 function effect transplant outcomes resulting in graft damage and eventual graft loss. We report several donor TLR4 polymorphisms that are associated with an increase in liver graft failure in liver allograft recipients.

Methods

Research subjects

A total of 738 liver donors were identified for this analysis. Lymph nodes or blood specimens were obtained by organ procurement organizations (OPOs) in the United States from deceased donors whose family or next of kin have given a generalized consent for research prior to organ and tissue donation. These OPOs included LifeSource in St. Paul, Minnesota, LifeQuest in Gainesville, Florida, New Jersey Organ & Tissue Sharing Network in New Providence, New Jersey, Organ Donor Center of Hawaii in Honolulu, Hawaii, Southwest Transplant Alliance in Dallas, Texas, One Legacy, California in Los Angeles, California, New England Organ Bank in Waltham, Massachusetts, Lifebanc in Cleveland, Ohio and Louisiana Organ Procurement Agency in Metairie, Louisiana. DNA was extracted from donor tissues using general laboratory methods.

Clinical data (Table 1) was obtained from the United Network for Organ Sharing (UNOS) via the Scientific Registry of Transplant Recipients (SRTR). The SRTR data system includes data on all donors, wait-listed candidates, and transplant recipients in the US, submitted by the members of the Organ Procurement and Transplantation Network (OPTN),

and has been described elsewhere (22). The Health Resources and Services Administration (HRSA), US Department of Health and Human Services, provides oversight to the activities of the OPTN and SRTR contractors.

Genotyping

Forty SNPs covering the entire TLR4 region were genotyped in DNA from liver donors. Genotyped SNPs were selected by SeattleSNPs (<http://pga.gs.washington.edu>). Selection was done to insure that all haplotype blocks within the TLR4 gene were represented in both Caucasian and African American samples. The selected SNPs were submitted to Illumina for processing by Illumina Assay Design Tool to create a panel of SNPs for genotyping. A total of 250 ng of genomic DNA per donor was used for Illumina SNP genotyping using the Illumina BeadArray platform following the manufacturer's protocol. Raw hybridization intensity data processing, clustering and genotype calling were performed using the genotyping module in the BeadStudio package (Illumina, San Diego, CA, USA). Before genotype calling, the trimmed mean intensities were calculated from the normalized intensity values obtained for each bead type on the array by rejecting outliers to ensure high quality of genotype data. Genotype calls were generated using the GenCall software incorporated into the BeadStudio package.

Statistical analysis

To examine the relationship between recipient and donor variables and all-cause liver graft failure (LGF), univariable Cox proportional hazards models were created. To identify genetic variants associated with LGF, we performed Cox regression on time to LGF for each genotyped SNP adjusting for donor and recipient race. Subgroup analysis was also done using Caucasian and African American donor samples separately. SNPs were coded for the additive genetic model. Correlation between transplant recipients with the same donor was accounted for using a robust sandwich estimator. The Bonferroni method was used to adjust the alpha-level for multiple comparisons in the overall analysis, and separately for each subgroup analysis. The family-wise Type I error rate for a two-sided significance test was taken to be 0.05.

A multivariable model was created by backwards selection with a retention p-value of 0.10. Potential covariates included: recipient race, HCV status, gender, age (linear and quadratic), BMI (linear and quadratic), MELD/PELD (linear and quadratic), cold ischemic time, first transplant, primary cause of liver disease, previous abdominal surgery, portal vein thrombosis, partial/split transplant, donor race, gender, age (linear and quadratic), height (linear and quadratic), donation after cardiac death (DCD), cause of donor death, and Liver Donor Risk Index (linear and quadratic). Recipient and donor race were forced into the model at all stages in the selection procedure. The covariates selected by backwards selection were included in the Cox regression model, and association between SNP and time to LGF was re-examined. All statistical analyses were conducted using SASv9.2 (The SAS Institute, Cary, NC, USA, <http://www.sas.com>).

Results

Clinical factors associated with LGF were determined in 738 recipients transplanted from 2006 to 2010 with a total of 139 (19%) all cause liver graft failures over a median (IQR) follow up of 13.0 (9.4–25.0) months post transplant. Analysis based on the specific cause of liver graft failure could not be done because the SRTR database does not collect detailed data on causes of graft loss and all the outcome data for this analysis is from SRTR. When stratified by race, 610 recipients received livers from Caucasian donors with 112 graft failures (18%) and 128 recipients received livers from African American donors with 27

graft failures (21%). Using univariable Cox regression, clinical factors of recipients that were associated with LGF included: HCV positive ($p = 0.038$), and higher PELD score ($p = 0.0085$) though the number of pediatric recipients was low ($n = 23$) (Table 1). Though both cold ischemia and warm ischemia time have been shown to be important variables in regards to liver graft failure the SRTR database does not reliably collect information on warm ischemia time and therefore was not listed separately in the Cox model. Clinical factors of donors that were significantly associated with LGF included the Liver Donor Risk Index ($p = 0.0028$) and age at organ recovery ($p=0.05$).

A total of 40 donor SNPs within the TLR4 gene were analyzed in all 738 liver allograft recipients for their association with LGF. Cox proportional hazards models were used, adjusting for recipient and donor race. Three SNPs, rs1927912, rs2737196 and rs1927910, were mono allelic in our population and were excluded from further analysis. Two SNPs exhibited a significant association with LGF after taking into account multiple comparisons ($\alpha = 0.05/37$ SNPs = 0.00135) and correcting for recipient race (Table 2). A SNP in the 5' promoter region of TLR4 (rs11536865, c.-728G>C) showed a significant association with LGF (HR=2.5, $p=0.0003$) though the minor allele frequency (MAF) was low (MAF=0.02) and a SNP in intron 2 (rs5030717, c.261-833A>G) showed a significant association with LGF (HR=1.67, $p=0.00079$). Two additional SNPs in the TLR4 gene also exhibited a modest association including rs913930 (HR=1.48, $p=0.0026$) located 3' of the TLR4 coding region and rs2770146 (HR=1.45, $p=0.0044$) located in intron 2 (c.261-1329T>C).

Subgroup analyses were also conducted by donor race. In Caucasian liver donors, SNP rs913930 was associated with an increased hazard of LGF (HR=1.62, $p=0.00055$) but not in African American donors (HR=0.78, $p=0.61$). Haplotype block analysis showed that the region between rs91390 and the coding sequences of TLR4 exhibits increased recombination in African Americans and thus would have lower linkage disequilibrium (LD) between SNPs in African Americans than in Caucasians. A Kaplan-Meier Curve showing time to LGF by genotype of rs91390 is shown in Figure 1. Graft survival among recipients with livers from Caucasian donors was significantly reduced for the T/T donor genotype versus the C/C genotype (HR=2.66, 95% CI (1.59, 4.45), $p=0.0002$). None of the SNPs were significant in recipients of livers from African American donors, though two variants, (rs11536865 and rs5030717) had suggestive p values ($p=0.0021$ and 0.0024 respectively) when multiple testing is taken into consideration. It should be noted that the number of African American liver donors genotyped is much smaller than Caucasian liver donors (128 vs. 610). Analysis of the SNPs using a multi-SNP model using the top three SNPs (rs91390, rs11536865 and rs5030717) showed that the association became less significant revealing that there is no strong haplotype effect.

A multivariable Cox proportional hazards model for LGF was also created (Table 3). The model was adjusted for recipient race, donor race (Caucasian and African American donors only), recipient HCV status, first liver transplant, previous abdominal surgery, recipient BMI (linear and quadratic), and Liver Donor Risk Index (linear). Liver Donor Risk Index had the most significant effect on LGF ($p=0.00031$) which has been previously associated with liver graft failure (23). Other variables of modest significance were recipient HCV status ($p=0.03$), first liver transplant ($p=0.036$), and recipient BMI (quadratic) ($p=0.0018$).

We also included SNPs in our multivariable Cox proportional hazards model for LGF. None of the SNPs remained significant after accounting for multiple comparisons using the Bonferroni correction. However SNPs rs11536865 ($p=0.00137$) and rs5030717 ($p=0.00282$) show suggestive evidence for association with LGF. We obtained similar results after removal of pediatric recipients ($n = 23$).

Discussion

We identified three SNPs within the deceased donor TLR4 gene that are significantly associated with LGF after taking multiple comparisons into account; rs11536856 and rs5030717 in all donors and rs913930 only in Caucasian donors. For all three SNPs, the risk for graft loss is increased when the minor allele is present. The Kaplan Meyer plot (Figure 1) shows that for rs913930 in Caucasian donors, the risk increases with an increasing number of minor alleles. It is of note that SNP rs913930, located in the 3' end of TLR4, is significant in Caucasians but not in African American donors. It is likely that rs913930 is not functional but is in LD with a functional SNP closer to or within the TLR4 gene. Haplotype block analysis showed that the region between this SNP and the TLR4 gene has a greater level of recombination in African Americans than in Caucasians. In the Caucasian liver donors LD, between rs913930 and the putative functional SNP, is maintained but LD is likely abolished in the African American donors due to more frequent recombination. Previously reported functional variants, SNPs rs4986790 (p.Asp299Gly) and rs4986791 (p.Thr399Ile), were not associated with LGF ($p=0.49$ and 0.95 , respectively) in our data. It may be that their contribution to graft failure is minimal, or not associated with the donor genotype or our study is under powered, or the reported associations were false positive.

Those SNPs identified in this study were associated with an increase in LGF. Possible mechanisms for an increase risk of graft loss could be a variant that either increases expression of TLR4 or increases mRNA stability resulting in a higher level of TLR4 signaling of inflammation, resulting in organ damage and eventual loss.

The SNP rs10759930 was modestly associated with HCV infection ($p=0.052$) in Caucasian donors but the importance of this is unknown. It has been previously shown that HCV status does not alter hepatic expression levels of TLR4 (13) but the p.Arg753Gln polymorphism has been associated with allograft failure after liver transplantation for chronic HCV (24).

Our study has several limitations. The SRTR does not provide detailed information about the cause of LGF therefore it is not possible to determine whether infectious complications were involved in development of LGF. The SRTR also does not provide information about the recurrence of hepatitis C after liver transplantation. Therefore it is not possible to determine the role of TLR4 SNPs in occurrence of infectious complications or in recurrence on hepatitis C.

Much work has been done on the identification of recipient genetic variants on allograft outcome but genetic variation in the donor genome will also most likely affect the outcome of the graft. Donor polymorphisms in TLR4 could act by modulating TLR4 activity and therefore affect the risk of graft loss. Additionally, there is a suggestion for an interaction between TLR4 SNPs and HCV status.

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Figure 1. Time to all-cause liver graft failure

Kaplan-Meier curve showing the effect of rs913930 genotypes on graft survival in Caucasians only. 610 recipients received livers from Caucasian donors with 112 (18%) having liver graft failure.

Table 1**Clinical and Demographic Features of Recipients and Donors**

Recipient Factors				
Variable	All (n=738)	No LGF (n=599)	LGF (n=139)	p-value
Age at Transplant	51.1 ± 15.0	50.6 ± 15.4	53.1 ± 13.4	0.098
Male Gender	499 (67.6%)	401 (66.9%)	98 (70.5%)	0.50
Race				0.22
African American	72 (9.8%)	57 (9.5%)	15 (10.8%)	
Caucasian	551 (74.7%)	452 (75.5%)	99 (71.2%)	
Other	115 (15.6%)	90 (15.0%)	25 (18.0%)	
HCV Positive	269 (39.0%)	210 (37.2%)	59 (47.2%)	0.038
HBV Core Antibody Positive	141 (20.5%)	113 (20.2%)	28 (21.7%)	0.24
Total Cold Ischemic Time (Hours)	6.5 ± 3.3	6.4 ± 3.3	6.8 ± 3.3	0.54
Primary Cause of Disease				0.076
Acute Hepatic Necrosis	35 (4.7%)	29 (4.8%)	6 (4.3%)	
HCV	151 (20.5%)	113 (18.9%)	38 (27.3%)	
Alcoholic Liver Disease	122 (16.5%)	106 (17.7%)	16 (11.5%)	
Cholestatic Disease	69 (9.4%)	61 (10.2%)	8 (5.8%)	
Metabolic Liver Disease	19 (2.6%)	17 (2.8%)	2 (1.4%)	
Malignancy	163 (22.1%)	134 (22.4%)	29 (20.9%)	
Other	179 (24.2%)	139 (23.2%)	40 (28.8%)	
MELD (n=680)	6221.6 ± 9.5	6221.4 ± 9.3	6222.2 ± 10.1	0.37
PELD (n=23)	6212.4 ± 11.7	6211.9 ± 11.8	6217.5 ± 13.4	0.0085

Donor Factors				
Variable	All (n=738)	No LGF (n=599)	LGF (n=139)	p-value
Age at Organ Recovery	40.0 ± 16.8	39.4 ± 16.7	42.6 ± 17.4	0.050
Male Gender	460 (62.3%)	372 (62.1%)	88 (63.3%)	0.96
Race				0.21
African American	128 (17.3%)	101 (16.9%)	27 (19.4%)	
Caucasian	610 (82.7%)	498 (83.1%)	112 (80.6%)	
Height, cm	171.6 ± 16.4	171.5 ± 16.8	171.9 ± 14.4	0.96
Cause of Death				0.15
Anoxia	149 (20.2%)	120 (20.0%)	29 (20.9%)	
Cerebrovascular/Stroke	283 (38.4%)	220 (36.7%)	63 (45.3%)	
Head Trauma	280 (37.9%)	237 (39.6%)	43 (30.9%)	
CNS Tumor	3 (0.4%)	2 (0.3%)	1 (0.7%)	
Other	23 (3.1%)	20 (3.3%)	3 (2.2%)	
DCD	43 (5.8%)	29 (4.8%)	14 (10.1%)	0.082
Liver Donor Risk Index	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	0.0028
Partial/Split Tx	22 (3.0%)	19 (3.2%)	3 (2.2%)	0.73
HCV Positive	22 (3.0%)	20 (3.3%)	2 (1.4%)	0.40

Table 2

TLR4 polymorphisms tested in all, Caucasian and African American liver donors

SNP	Location*	All donor Genotypes			Caucasian donor Genotypes			African American donor Genotypes		
		p-value	Allele	MAF	HR	p-value	Allele	MAF	HR	p-value
rs10759930	5' (-5130)	0.060158	T	0.36	0.78	0.058631	T	0.38	0.77	0.772295
rs16906053	5' (-4569)	0.266663	C	0.03	0.63	Mono	T	0	-	0.239314
rs2737191	5' (-4036)	0.009493	G	0.26	1.41	0.015135	G	0.29	1.41	0.247597
rs2770150	5' (-3612)	0.009493	G	0.26	1.41	0.015135	G	0.29	1.41	0.247597
rs2737190	5' (-2570)	0.558795	G	0.38	0.93	0.739754	G	0.32	0.96	0.382784
rs10116253	5' (-2431)	0.794221	C	0.27	1.03	0.773095	C	0.26	0.96	0.286083
rs1927914	5' (-2026)	0.559366	G	0.38	0.93	0.740955	G	0.32	0.96	0.382784
rs10759932	5' (-1607)	0.102705	C	0.15	1.28	0.32826	C	0.14	1.2	0.150727
rs11536865	5' (-728)	0.000299	C	0.02	2.50	Mono	G	0	-	0.002049
rs7864330	Intron 1	0.581251	G	0.05	0.85	0.393433	G	0.05	0.73	0.856491
rs1927911	Intron 1	0.603751	A	0.31	0.94	0.734631	A	0.26	0.95	0.472596
rs1927907	Intron 2	0.237139	A	0.15	1.21	0.388042	A	0.14	1.17	0.505385
rs10983756	Intron 2	0.950394	T	0.05	0.98	0.419721	T	0.05	0.74	0.214308
rs2770146	Intron 2	0.004443	C	0.28	1.45	0.006603	C	0.31	1.46	0.247597
rs5030717	Intron 2	0.000790	G	0.11	1.67	0.066508	G	0.1	1.44	0.002406
rs5030710	p.105Ser/Ser	0.49016	C	0.02	0.14	Mono	T	0	-	0.050944
rs4986790	p.Asp299Gly	0.431594	G	0.05	0.78	0.584098	G	0.05	0.82	0.676704
rs4986791	p.Thr399Ile	0.799131	T	0.04	0.92	0.735692	T	0.05	0.9	0.706622
rs5030718	p.Glu474Lys	0.765995	A	0.01	1.24	Mono	G	0	-	0.804558
rs7869402	3' UTR (1106)	0.452829	T	0.06	0.81	0.185779	T	0.04	0.56	0.995937
rs11536889	3' UTR (1205)	0.049893	C	0.14	0.67	0.062323	C	0.16	0.67	0.59956
rs7873784	3' UTR (2010)	0.253629	C	0.15	0.82	0.48449	C	0.15	0.89	0.320225
rs11536897	(3084)	0.434765	A	0.05	0.79	0.198081	A	0.05	0.67	0.59139
rs1927906	(3189)	0.917543	C	0.13	1.02	0.2158	C	0.1	0.7	0.116237
rs11536898	(3284)	0.190961	A	0.13	0.76	0.334226	A	0.13	0.81	0.34868
rs1554973	(3886)	0.312126	C	0.29	0.87	0.160602	C	0.25	0.8	0.815699
rs7044464	(4471)	0.316627	A	0.15	0.84	0.48449	A	0.15	0.89	0.450592
rs7856729	(4930)	0.620491	T	0.16	0.93	0.471092	T	0.15	0.88	0.887305

SNP	Location [*]	All donor Genotypes			Caucasian donor Genotypes			African American donor Genotypes					
		p-value	Allele	MAF	HR	p-value	Allele	MAF	HR	p-value	Allele	MAF	HR
rs7846989	(6014)	0.494820	C	0.12	0.87	0.221169	C	0.09	0.71	0.730267	C	0.33	1.12
rs7860896	(6177)	0.441845	G	0.13	0.86	0.223799	G	0.09	0.71	0.928129	G	0.38	1.03
rs7037117	(6737)	0.271190	G	0.31	0.86	0.139669	G	0.26	0.79	0.782831	A	0.35	1.1
rs913930	(7083)	0.002622	G	0.31	1.48	0.000552	G	0.36	1.62	0.615075	G	0.17	0.78
rs1330305	(7233)	0.041406	C	0.00	2.35	Mono	T	0	-	0.31408	C	0.02	1.84
rs1927905	(8382)	0.918929	C	0.07	0.98	0.187758	C	0.06	0.67	0.199636	C	0.17	1.44
rs7045953	(8869)	0.567126	G	0.17	0.92	0.471092	G	0.15	0.88	0.986548	G	0.26	1.01
rs7020005	(10141)	0.827684	T	0.01	0.89	Mono	C	0	-	0.933921	T	0.06	0.96
rs10759934	(12070)	0.028697	A	0.47	0.75	0.033545	A	0.49	0.75	0.561349	T	0.19	0.79

* Nucleotide numbers are from the ATG translational start site for 5' SNPs and from the translational stop site for 3' SNPs

Mono – mono allelic

MAF – Minor allele frequency

HR – Hazard ratio

Table 3

Cox proportional Hazard Model for all-cause Liver Graft Failure

Variable	Group	Reference Group	Hazard Ratio	HR Lower CL	HR Upper CL	P-value	Type 3 P-value
Donor Race	African American	Caucasian	0.949	0.582	1.547	0.83	0.83
Recipient Race	African American	Caucasian	1.207	0.652	2.232	0.55	0.41
Recipient Race	other	Caucasian	1.361	0.847	2.186	0.20	.
Recipient HCV Status	positive	negative	1.525	1.036	2.244	0.032	0.032
First Liver Tx	no	yes	1.917	1.043	3.526	0.036	0.036
Previous Abdominal Surgery	yes	no	1.500	1.011	2.224	0.044	0.044
Recipient BMI (linear)			1.020	0.982	1.059	0.31	0.31
Recipient BMI (quadratic)			0.993	0.988	0.997	0.0018	0.0018
Liver Donor Risk Index (linear)			2.559	1.537	4.261	0.00031	0.00031